# DESCRIPTION

## GENES FOR INCREASING CROP YIELD AND USES THEREOF

### Technical Field

The present invention relates to isolation and identification of genes that regulate the increase and decrease of the particle-bearing number (including glumous flowers, fruits, and seeds) of plants, and breeding methods that utilize these genes to increase the particle-bearing number (including glumous flowers, fruits, and seeds) of plants.

### Background Art

As the world population continues to show explosive growth, arable land is rapidly decreasing due to environmental pollution, global warming, and desertification, and chronic food shortage persists mainly in developing countries. Furthermore, the present grain production growth rate of 1.0% is low compared to the annual global population growth rate of 1.4%; furthermore, in the year 2025, when the world population is predicted to exceed eight billion, grain requirements will increase by 50%, further accelerating food shortage. In order to break this serious situation, not only political and economic measures, but also scientific grain breeding strategies that will increase the amount of grain production are necessary. This serious condition can no longer be avoided by conventional breeding, using cross-fertilization and selection techniques alone, and studies on grain plant type aimed at increasing yield, as well as specific and efficient grain breeding are necessary.

When concern was raised on world food crisis in the 1960's, short-culm high-yielding rice called miracle rice was cultivated at the International Rice Research Institute (Philippines), and short-culm high-yielding wheat was cultivated at the International Maize and Wheat Improvement Center (Mexico). World food crisis was avoided due to the rapid spread of both of these varieties. This is the so-called "Green Revolution". Both varieties showed twice the yield of conventional varieties, and this high-yielding character was caused by a short-culm plant type called a semi-dwarf. However, while application of nitrogenous fertilizers is necessary when aiming for a high yield, this simultaneously induces elongation of the plant, and lodging of the elongated grain by rain and wind dramatically decreases yield. On the other hand, even when fertilizers are applied, short-culm varieties allow an increased yield without succulent growth. That is, both short-culm varieties that contributed to the "Green Revolution" dramatically increased the yield by acquiring lodging resistance. At present, semi-dwarfism of grains greatly contributes to increased yield, but since many grain varieties already utilize semi-dwarf genes, further increase in yield using this technique cannot be expected, and

development of grain production technologies that utilize new techniques is necessary.

Rice is utilized as food by 50% of the world's human population. In particular, for those living in Asia, its cultivation characteristics match the highly humid monsoon climate, and not only has it been the source of energy for a long time as the staple diet, but it has also been well established in life and culture. Accordingly, breeding has been carried out in many places, and it has been improved to have characteristics that are convenient for use by humankind. Furthermore, with the recent determination of the genomic nucleotide sequence of rice, tools for molecular genetics are being put into place, and development of new breeding technology using genomic genetics is expected.

To date, various attempts have been made to increase the yield of grains (plants); however, there are no reports of isolation and identification of genes relating to the increase and decrease of the number of flowers and seeds (glumous flowers) that are directly responsible for increase in yield. In addition to the conventional semi-dwarfing of grains, if techniques to regulate the increase and decrease in the number of flowers and seeds (glumous flowers) are developed, further increase in grain yield can be expected.

Disclosure of the Invention

The present invention has been made in view of the above circumstances. An objective of this invention is to isolate and identify genes relating to the increase and decrease of the particle-bearing number (including glumous flowers, fruits, and seeds) of plants, and to provide breeding methods for increasing the particle-bearing number (including glumous flowers, fruits, and seeds) of plants, using these genes.

In an attempt to increase the grain (plant) yield, the present inventors used a rice plant, which serves as a model for a monocotyledonous plant, to search for genes that are directly responsible for increase in yield, more specifically, for genes relating to the increase and decrease of the number of flowers and seeds (glumous flowers). The number of flowers and seeds (glumous flowers) is controlled as a quantitative trait (QTL) by the interaction of more than one gene. Therefore, in order to generate a hybrid population for QTL analysis, parent varieties of the hybrids were selected. Two varieties showing a clear difference in the particle-bearing number, "Koshihikari" which is a japonica rice, and "Habataki" which is an indica rice, were selected (Fig. 1). F1 individuals produced by crossing these two varieties were backcrossed using Koshihikari as the repeated parent and self-fertilization was carried out. The resulting 74 varieties of BC2F1, BC2F2, and BC3F2 populations were cultivated and developed on the Nagoya University Farm. By performing a QTL analysis relating to the particle-bearing number using 74 BC2F2 plants, a plurality of QTL that increase the particle-bearing number was detected (Fig. 2). In particular, a QTL (YQ1; Yielding QTL 1) of

Habataki, located at approximately 28 cM in the short arm of chromosome 1 (Fig. 2), was successfully discovered to be very effective in increasing the grain (or seed) number as compared to that of Koshihikari. To verify the presence of YQ1, repeated backcrossing and MAS were used to produce a YQ1 semi-isogenic line, and the maximum particle-bearing numbers of Nil-YQ1 and Koshihikari (control) were investigated. As a result, the presence of QTL(YQ1) was confirmed, and varieties in which the locus at approximately 28 cM in the short arm of chromosome 1 was substituted with that of Habataki were found to increase their particle-bearing number by an average of 50.

Next, from each of the 74 BC2F1 individuals, DNAs were extracted using the CTAB method, and the genotype of each individual was determined using 93 molecular markers that thoroughly cover all chromosomes. Their self-fertilized progeny, BC2F2, was developed at ten individuals per variety, and from among them, one individual per variety was randomly selected. After sampling six panicles from each of the selected individuals, the particle-bearing number was investigated for each panicle. Among the six panicles of each variety, the panicle having the largest particle-bearing number was selected, and this number was used as the maximum particle-bearing number. QTL analysis was then performed using the Qgene software.

The BC3F2 population was then used to investigate the phenotype and genotype (F2 and F3) using molecular markers, and linkage analysis was performed again. The results showed that YQ1 is located between molecular markers 6A and 8A (Fig. 3). As a result of high-resolution linkage analysis using a segregating population (12500 individuals) of YQ, the YQ1 locus was specified to be in a region of approximately 8 Kb between molecular markers 4A9 and 20 (Fig. 4). When gene prediction was performed in this region, a single gene was predicted, and, as a result of a homology search, the gene was found to be highly homologous to CKX (cytokinin oxidase) (Fig. 4). When the nucleotide sequence of this CKX gene was determined for Habataki and Koshihikari, differences in the nucleotides were found, and the CKX of Habataki seemed to have lost its function (Fig. 5).

Furthermore, when the rice genomic sequence was searched to analyze the rice CKX gene, eleven CKX genes were found to be present in the rice genome. When a phylogenic tree was constructed for these genes as well as the CKX genes in *Arabidopsis thaliana*, AtCKX2, 3, and 4 of *Arabidopsis thaliana*, and five rice CKX genes (CKX located on Chr.1 25 cM P695A4, CKX located on Chr.127 P419B01 (the present gene), CKX gene located on Chr.6 79 cM OsJ0006A22-GS, and two CKX genes located on Chr.2 32 cM) were found to be very closely related (Fig. 6). When the homologies of these genes were investigated, they were found to be highly homologous at the amino acid level (Figs. 7 to 9). Furthermore, when the locus positions of all CKX genes in rice were confirmed, some of them were found to be located on the YQ regions (Fig. 10).

More specifically, the present inventors succeeded in isolating a novel gene involved in the increase and decrease of the particle-bearing number of plants, and thus completed this invention.

The present invention relates to the isolation and identification of genes that regulate the increase and decrease of the particle-bearing number (including glumous flowers, fruits, and seeds) of plants, and breeding methods that utilize these genes to increase the particle-bearing number (including glumous flowers, fruits, and seeds) of plants, and this invention provides [1] to [19] described below.

[1] A DNA encoding a plant-derived protein whose deletion of function causes an increase in the particle-bearing number of a plant, wherein the DNA is any one of (a) to (d):

(a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 3;

(b) a DNA comprising a coding region comprising the nucleotide sequence of SEQ ID NO: 1 or 2;

(c) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 3, wherein one or more amino acids have been substituted, deleted, added, and/or inserted; and

(d) a DNA that hybridizes under stringent conditions with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 2.

[2] The DNA of [1], wherein the DNA is derived from rice.

[3] A DNA encoding an RNA complementary to a transcript of the DNA of [1] or [2].

[4] A DNA encoding an RNA having ribozyme activity that specifically cleaves a transcript of the DNA of [1] or [2].

[5] A DNA encoding an RNA that suppresses the expression of the DNA of [1] or [2] by cosuppression effects at the time of expression in plant cells.

[6] A vector comprising the DNA of any one of [1] to [5].

[7] A host cell transfected with the vector of [6].

[8] A plant cell transfected with the vector of [6].

[9] A transformed plant comprising the plant cell of [8].

[10] A transformed plant that is an offspring or a clone of the transformed plant of [9].

[11] A reproductive material of the transformed plant of [9] or [10].

[12] A method for producing a transformed plant, wherein the method comprises the steps of introducing the DNA of any one of [1] to [5] into a plant cell, and regenerating a plant body from said plant cell.

[13] A protein encoded by the DNA of [1] or [2].

[14] A method for producing the protein of [13], wherein the method comprises the steps of culturing the host cell of [7], and collecting a recombinant protein from said cell or from a culture supernatant thereof.

[15] An antibody that binds to the protein of [13].

[16] A polynucleotide comprising at least 15 continuous nucleotides that are complementary to the nucleotide sequence of SEQ ID NO: 1 or 2, or a complementary sequence thereof.

[17] A method for increasing the particle-bearing number of a plant, wherein the method comprises the step of expressing the DNA of any one of [3] to [5] in the cells of a plant body.

[18] An agent for changing the particle-bearing number of a plant, wherein the agent comprises the DNA of any one of [1] to [5] or the vector of [6] as an active ingredient.

[19] A method for determining the particle-bearing number of a plant, wherein the method comprises the steps of:

(a) preparing a DNA sample from a test plant body, or a reproductive medium thereof;

(b) amplifying a region of said DNA sample corresponding to the DNA of [1]; and

(c) determining the nucleotide sequence of the amplified DNA region;

wherein the plant is determined to be a variety having a small particle-bearing number when the nucleotide sequence encodes a protein whose deletion of function causes an increase in the particle-bearing number of a plant, and the plant is determined to be a variety having a large particle-bearing number when said protein is not encoded.

The present invention provides DNAs that encode rice-derived CKX proteins. The genomic sequence of the DNA in "Koshihikari" is shown in SEQ ID NO: 1, its cDNA sequence is shown in SEQ ID NO: 2, and the amino acid sequence of a protein encoded by this DNA is shown in SEQ ID NO: 3. The genomic sequence of the DNA in "Habataki" is shown in SEQ ID NO: 4, its cDNA sequence is shown in SEQ ID NO: 5, and the amino acid sequence of a protein encoded by this DNA is shown in SEQ ID NO: 6.

The CKX gene isolated by the present invention is located at one of the quantitative trait loci (QTL) that were detected by utilizing the crossed progeny of "Habataki" and "Koshihikari", and was found to be located on chromosome 1. When the nucleotide sequence of this CKX gene was determined in Habataki and Koshihikari, differences in the nucleotides were found, and the CKX protein of Habataki, which has a larger particle-bearing number as compared to other varieties such as Koshihikari, was found to have lost its function.

Cytokinin (a general term for a group of compounds having biological activity similar to that of kinetin, and which have a substituent at position 6 of adenine), which is a phytohormone, is involved in promoting cell division, flower bud formation, lateral bud formation, suppression of aging, stomatal movement, root elongation, and such. In particular, promotion of flower bud formation and lateral bud formation may be closely linked to the trait of interest (increase in the number of glumous flowers). Using mevalonic acid as the substrate, cytokinin is synthesized via four catalytic reactions, but it is inactivated by cleavage at position 6 of adenine by cytokinin

oxidase (for example, zeatin is degraded into adenine and methylbutenal). More specifically, when the function of the CKX (cytokinin oxidase) gene is lost, cytokinin cannot be degraded, and as a result, cytokinin accumulates. Since accumulation of cytokinin induces flower bud formation, this may lead to an increase in the particle-bearing number (number of glumous flowers), which agrees well with the function and phenotype of the CKX gene. This indicates that deletion of the function of the CKX gene increases the particle-bearing number (number of glumous flowers) of rice plants, and, as a result, the yield is increased. So far, a gene considered to be linked to the increase in particle-bearing number (number of glumous flowers) has neither been identified nor isolated. By proceeding through complicated steps, the present inventors finally elucidated the region where it exists and succeeded in isolating the gene as a single gene for the first time.

At present, increasing the particle-bearing number is an important breeding objective in Japanese rice variety improvement. Increasing the particle-bearing number directly leads to a trait that increases the grain yield, and since such traits are agriculturally very important, they are expected to be applied to breeding through the use of the CKX gene.

Since deletion of the function of the CKX gene increases the particle-bearing number of the plant, regulating the expression of this DNA using the antisense method, ribozyme method, and such can result in increasing the yield of grains. For example, by introducing the CKX gene in the antisense direction to a variety whose CKX gene is functioning, such as "Koshihikari", the particle-bearing number can be increased. Furthermore, the particle-bearing number can be increased by introducing an inactivated form of the CKX gene using molecular markers. The method of introduction may be transformation or crossing. The period of time required for transformation is very short as compared to gene transfer by crossing, and this allows the particle-bearing number to be increased without accompanying changes in other traits. The use of the CKX gene, which was isolated in this invention and relates to the increase and decrease of particle-bearing number, allows the particle-bearing number of rice plants to be changed easily, and may contribute to the cultivation of rice varieties whose particle-bearing number is increased. Since genomic synteny (genetic homology) is very highly conserved among grains, application of the rice CKX gene in breeding grains such as wheat, barley, and corn can be expected. Furthermore, since the CKX gene is not limited to grains and is widely distributed among plants, deletion of the CKX gene function may increase the number of flowers and seeds (glumous flowers) in all plants, leading to increased yield.

DNA encoding the CKX protein of the present invention include genomic DNA, cDNA, and chemically synthesized DNA. A genomic DNA and cDNA can be prepared according to conventional methods known to those skilled in the art. More specifically, a genomic DNA can be prepared, for example, as follows: (1) extract genomic DNA from rice varieties having the

CKX gene (*e.g.* Koshihikari); (2) construct a genomic library (utilizing a vector, such as plasmid, phage, cosmid, BAC, and PAC); (3) spread the library; and (4) conduct colony hybridization or plaque hybridization using a probe prepared based on the DNA encoding a protein of the present invention (*e.g.* SEQ ID NO: 1 or 2). Alternatively, a genomic DNA can be prepared by PCR, using primers specific to a DNA encoding the protein of s present invention (*e.g.* SEQ ID NO: 1 or 2). On the other hand, cDNA can be prepared, for example, as follows: (1) synthesize cDNAs based on mRNAs extracted from rice varieties having the CKX gene (*e.g.* Koshihikari); (2) prepare a cDNA library by inserting the synthesized cDNA into vectors, such as λZAP; (3) spread the cDNA library; and (4) conduct colony hybridization or plaque hybridization as described above. Alternatively, cDNA can be also prepared by PCR.

The present invention includes DNAs encoding proteins (Koshihikari) functionally equivalent to the CKX protein of SEQ ID NO: 3. Herein, the term "functionally equivalent to the CKX protein" indicates that deletion of function of the object protein results in an increase in the particle-bearing number. Such DNA is derived preferably from monocotyledonous plants, more preferably from Gramineae, and most preferably from rice.

Examples of such DNAs include those encoding mutants, derivatives, alleles, variants, and homologues comprising the amino acid sequence of SEQ ID NO: 3 wherein one or more amino acids are substituted, deleted, added and/or inserted.

Examples of methods for preparing a DNA encoding a protein comprising altered amino acids well known to those skilled in the art include the site-directed mutagenesis (Kramer, W. and Fritz, H. -J., (1987) "Oligonucleotide-directed construction of mutagenesis via gapped duplex DNA." Methods in Enzymology, 154: 350-367). The amino acid sequence of a protein may also be mutated in nature due to the mutation of a nucleotide sequence. A DNA encoding proteins having the amino acid sequence of a natural CKX protein wherein one or more amino acids are substituted, deleted, and/or added are also included in the DNA of the present invention, so long as they encode a protein functionally equivalent to the natural CKX protein (SEQ ID NO: 3). Additionally, nucleotide sequence mutants that do not give rise to amino acid sequence changes in the protein (degeneracy mutants) are also included in the DNA of the present invention.

A DNA encoding a protein functionally equivalent to the CKX protein described in SEQ ID NO: 3 can be produced, for example, by methods well known to those skilled in the art including: methods using hybridization techniques (Southern, E.M.: Journal of Molecular Biology, Vol. 98, 503, 1975.); and polymerase chain reaction (PCR) techniques (Saiki, R. K. *et al.* Science, vol.230, 1350-1354, 1985; Saiki, R. K. *et al.* Science, vol.239, 487-491, 1988). That is, it is routine for a person skilled in the art to isolate a DNA with high homology to the CKX gene from rice and other plants using the nucleotide sequence of the CKX gene (SEQ ID NO: 2)

or parts thereof as a probe, and oligonucleotides hybridizing specifically to the nucleotide sequence of CKX gene (SEQ ID NO: 2) as a primer. Such DNA encoding proteins functionally equivalent to the CKX protein, obtainable by hybridization techniques or PCR techniques, are included in the DNA of this invention.

Hybridization reactions to isolate such DNAs are preferably conducted under stringent conditions. Stringent hybridization conditions of the present invention include conditions such as: 6 M urea, 0.4% SDS, and 0.5x SSC; and those which yield a similar stringency to the conditions. DNAs with higher homology are expected when hybridization is performed under conditions with higher stringency, for example, 6 M urea, 0.4% SDS, and 0.1x SSC. Those DNAs isolated under such conditions are expected to encode a protein having a high amino acid level homology with CKX protein (SEQ ID NO: 3 or 6). Herein, high homology means an identity of at least 50% or more, more preferably 70% or more, and much more preferably 90% or more (*e.g.* 95%, 96%, 97%, 98%, 99%, or more), through the entire amino acid sequence. The degree of homology of one amino acid sequence or nucleotide sequence to another can be determined by following the algorithm BLAST by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990; Proc. Natl. Acad. Sci. USA, 90: 5873, 1993). Programs such as BLASTN and BLASTX were developed based on the BLAST algorithm (Altschul SF, *et al.* J. Mol. Biol.215: 403, 1990). To analyze a nucleotide sequences according to BLASTN, the parameters are set, for example, as score= 100 and word length= 12. On the other hand, parameters used for the analysis of amino acid sequences by the BLASTX include, for example, score= 50 and word length= 3. Default parameters of each program are used when using BLAST and Gapped BLAST program. Specific techniques for such analyses are known in the art.

Evaluation of whether a particular DNA encodes a protein relating to the increase and decrease of the particle-bearing number of a plant can be performed as follows. The most conventional method involves deleting the function of the DNA, then performing the cultivation, and investigating the particle-bearing number. More specifically, the method involves cultivating under conditions where the function of the DNA is maintained, and under conditions where the function of the DNA is deleted, and comparing the resulting particle-bearing numbers. If the particle-bearing numbers do not change or are nearly the same, the DNA is judged not to be involved in the increase and decrease of the particle-bearing number. When the DNA is involved in the increase and decrease of the particle-bearing number, the particle-bearing number is further increased, and this difference is considered to be the degree of increase or decrease of the particle-bearing number.

The DNA of the present invention can be used, for example, to prepare recombinant proteins, and to produce plant transformants having an altered particle-bearing number. A

recombinant protein is usually prepared by inserting a DNA encoding a protein of the present invention into an appropriate expression vector, introducing the vector into an appropriate cell, culturing the transformed cells, allowing the cells to express the recombinant protein, and purifying the expressed protein. A recombinant protein can be expressed as a fusion protein with other proteins so as to be easily purified, for example, as a fusion protein with maltose binding protein in *Escherichia coli* (New England Biolabs, USA, vector pMAL series), as a fusion protein with glutathione-S-transferase (GST) (Amersham Pharmacia Biotech, vector pGEX series), or tagged with histidine (Novagen, pET series). The host cell is not limited so long as the cell is suitable for expressing the recombinant protein. It is possible to utilize yeasts or various animal, plant, or insect cells besides the above described *E. coli.* A vector can be introduced into a host cell by a variety of methods known to one skilled in the art. For example, a transformation method using calcium ions (Mandel, M. and Higa, A. (1970) Journal of Molecular Biology, 53, 158-162, Hanahan, D. (1983) Journal of Molecular Biology, 166, 557-580) can be used to introduce a vector into *E. coli.* A recombinant protein expressed in host cells can be purified and recovered from the host cells or the culture supernatant thereof by known methods. When a recombinant protein is expressed as a fusion protein with maltose binding protein or other partners, the recombinant protein can be easily purified by affinity chromatography. A protein of the present invention can be prepared from transformed plants which have been generated by introducing a DNA of this invention into plants as described below. Thus, the transformed plants of the present invention include not only the plants harboring a DNA of this invention which has been introduced to alter the particle-bearing number as described below, but also the plants harboring a DNA of this invention which has been introduced to prepare a protein of this invention.

The resulting protein can be used to prepare an antibody that binds to the protein. For example, a polyclonal antibody can be prepared by immunizing immune animals, such as rabbits, with a purified protein of the present invention or a portion thereof, collecting blood after a certain period, and removing clots. A monoclonal antibody can be prepared by fusing myeloma cells with the antibody-forming cells of animals immunized with the above protein or its portion, isolating a monoclonal cell expressing a desired antibody (hybridoma), and recovering the antibody from the cell. The obtained antibody can be utilized to purify or detect a protein of the present invention. Accordingly, the present invention includes antibodies that bind to proteins of the invention. The use of these antibodies enables one to distinguish the expression site of proteins involved in the increase and decrease of the particle-bearing number of a plant body, or determine whether the plant species express the protein involved in the increase and decrease of the particle-bearing number.

For example, since an antibody that specifically recognizes the amino acid sequence of

the carboxyl terminus of "Koshihikari" having a small particle-bearing number does not bind to proteins expressed in varieties such as "Habataki" having a large particle-bearing number, it can be used to distinguish whether or not the protein involved in the increase and decrease of particle-bearing number is expressed in a particular plant species.

When producing a transformed plant in which the particle-bearing number has been increased by utilizing the DNA of this invention, a DNA for suppressing the expression of the DNA encoding the protein of this invention is inserted into an appropriate vector, which is then introduced into a plant cell. The transformed plant cells obtained by these steps are then regenerated. The plant cells to receive the vector are preferably plant cells that show normal expression of the DNA of this invention. "Suppressing the expression of the DNA encoding the protein of this invention" includes suppression of gene transcription and suppression of translation into the protein. Furthermore, it not only includes complete termination of DNA expression, but also includes decreased expression.

Suppression of the expression of a particular endogenous gene in a plant can be performed, for example by utilizing a DNA that encodes an RNA complementary to the transcript of the DNA encoding the protein of this invention.

One embodiment of "a DNA that encodes an RNA complementary to the transcript of the DNA encoding the protein of this invention" is a DNA that encodes an antisense RNA complementary to the transcript of the DNA encoding the protein of this invention. Ecker *et al.* were the first to demonstrate the antisense effect of an antisense RNA introduced by electroporation in plant cells by using the transient gene expression method (J. R. Ecker and R. W. Davis (1986) Proc. Natl. Acad. Sci. USA 83: 5372). Thereafter, the target gene expression was reportedly reduced in tobacco and petunias by expressing antisense RNAs (A. R. van der Krol *et al.* (1988) Nature 333: 866). The antisense technique has now been established as a means to repress target gene expression in plants.

Multiple factors cause antisense nucleic acids to repress the target gene expression. These include: inhibition of transcription initiation by triple strand formation; repression of transcription by hybrid formation at the site where the RNA polymerase has formed a local open loop structure; transcription inhibition by hybrid formation with the RNA being synthesized; repression of splicing by hybrid formation at the junction between an intron and an exon; repression of splicing by hybrid formation at the site of spliceosome formation; repression of mRNA translocation from the nucleus to the cytoplasm by hybrid formation with mRNA; repression of splicing by hybrid formation at the capping site or at the poly A addition site; repression of translation initiation by hybrid formation at the binding site for the translation initiation factors; repression of translation by hybrid formation at the site for ribosome binding near the initiation codon; inhibition of peptide chain elongation by hybrid formation in the

translated region or at the polysome binding sites of mRNA; and repression of gene expression by hybrid formation at the sites of interaction between nucleic acids and proteins. These factors repress the target gene expression by inhibiting the process of transcription, splicing, or translation (Hirashima and Inoue, "Shin Seikagaku Jikken Koza (New Biochemistry Experimentation Lectures) 2, Kakusan (Nucleic Acids) IV, Idenshi No Fukusei To Hatsugen (Replication and Expression of Genes)," Nihon Seikagakukai Hen (The Japanese Biochemical Society), Tokyo Kagaku Dozin, pp. 319-347, (1993)).

An antisense sequence of the present invention can repress the target gene expression by any of the above mechanisms. In one embodiment, if an antisense sequence is designed to be complementary to the untranslated region near the 5' end of the gene's mRNA, it will effectively inhibit translation of a gene. It is also possible to use sequences complementary to the coding regions or to the untranslated region on the 3' side. Thus, the antisense DNA used in the present invention include DNA having antisense sequences against both the untranslated regions and the translated regions of the gene. The antisense DNA to be used is connected downstream from an appropriate promoter, and, preferably, a sequence containing the transcription termination signal is connected on the 3' side. The DNA thus prepared can be transfected into the desired plant by known methods. The sequence of the antisense DNA is preferably a sequence complementary to the endogenous gene of the plant to be transformed or a part thereof, but it need not be perfectly complementary, so long as it can effectively inhibit the gene expression. The transcribed RNA is preferably at least 90%, and most preferably at least 95% complementary to the transcripts of the target gene. In order to effectively inhibit the expression of the target gene by means of an antisense sequence, the antisense DNA should be at least 15 nucleotides long, more preferably at least 100 nucleotides long, and still more preferably at least 500 nucleotides long. The antisense DNA to be used is generally shorter than 5 kb, and preferably shorter than 2.5 kb.

DNA encoding ribozymes can also be used to repress the expression of endogenous genes. A ribozyme is an RNA molecule that has catalytic activity. There are many known ribozymes having various activities. Research on ribozymes as RNA cleaving enzymes has enabled the design of a ribozyme that site-specifically cleaves RNA. While some ribozymes of the group I intron type or the M1RNA contained in RNaseP consist of 400 nucleotides or more, others belonging to the hammerhead type or the hairpin type have an activity domain of about 40 nucleotides (Makoto Koizumi and Eiko Ohtsuka (1990) Tanpakushitsu Kakusan Kohso (Protein, Nucleic acid and Enzyme) 35: 2191).

The self-cleavage domain of a hammerhead type ribozyme cleaves at the 3' side of C15 of the sequence G13U14C15. Formation of a nucleotide pair between U14 and A at the ninth position is considered important for the ribozyme activity. Furthermore, it has been shown that

the cleavage also occurs when the nucleotide at the 15th position is A or U instead of C (M. Koizumi *et al.* (1988) FEBS Lett. 228: 225). If the substrate binding site of the ribozyme is designed to be complementary to the RNA sequences adjacent to the target site, one can create a restriction-enzyme-like RNA cleaving ribozyme which recognizes the sequence UC, UU, or UA within the target RNA (M. Koizumi *et al.* (1988) FEBS Lett. 239: 285; Makoto Koizumi and Eiko Ohtsuka (1990) Tanpakushitsu Kakusan Kohso (Protein, Nucleic acid and Enzyme), 35: 2191; M. Koizumi *et al.* (1989) Nucleic Acids Res. 17: 7059). For example, in the coding region of the CKX gene (SEQ ID NO: 2), there are a number of sites that can be used as the ribozyme target.

The hairpin type ribozyme is also useful in the present invention. A hairpin type ribozyme can be found, for example, in the minus strand of the satellite RNA of the tobacco ringspot virus (J. M. Buzayan, Nature 323: 349 (1986)). This ribozyme has also been shown to target-specifically cleave RNA (Y. Kikuchi and N. Sasaki (1992) Nucleic Acids Res. 19: 6751; Yo Kikuchi (1992) Kagaku To Seibutsu (Chemistry and Biology) 30: 112).

The ribozyme designed to cleave the target is fused with a promoter, such as the cauliflower mosaic virus 35S promoter, and with a transcription termination sequence, so that it will be transcribed in plant cells. However, if extra sequences have been added to the 5' end or the 3' end of the transcribed RNA, the ribozyme activity can be lost. In this case, one can place an additional trimming ribozyme, which functions in cis to perform the trimming on the 5' or the 3' side of the ribozyme portion, in order to precisely cut the ribozyme portion from the transcribed RNA containing the ribozyme (K. Taira *et al.* (1990) Protein Eng. 3: 733; A. M. Dzaianott and J. J. Bujarski (1989) Proc. Natl. Acad. Sci. USA 86: 4823; C. A. Grosshands and R. T. Cech (1991) Nucleic Acids Res. 19: 3875; K. Taira *et al.* (1991) Nucleic Acid Res. 19: 5125). Multiple sites within the target gene can be cleaved by arranging these structural units in tandem to achieve greater effects (N. Yuyama *et al.*, Biochem. Biophys. Res. Commun. 186: 1271 (1992)). By using such ribozymes, it is possible to specifically cleave the transcripts of the target gene in the present invention, thereby repressing the expression of the gene.

Another embodiment of "a DNA that encodes an RNA complementary to the transcript of the DNA encoding the protein of this invention" is a DNA encoding a dsRNA complementary to the transcript of the DNA encoding the protein of this invention. RNAi is a phenomenon where introduction into cells of a double-stranded RNA (hereinafter, dsRNA) comprising a sequence identical or similar to a target genetic sequence suppresses the expression of both the introduced foreign gene and the endogenous target gene. When approximately 40 to several hundred base pairs of dsRNA are introduced into cells, an RNaseIII-like nuclease called Dicer, which has a helicase domain, excises the dsRNA in the presence of ATP from the 3' end, approximately 21 to 23 base pairs at a time, and produces siRNA (short interference RNA).

Binding of a specific protein to this siRNA forms a nuclease complex (RISC: RNA-induced silencing complex). This complex recognizes and binds a sequence that is the same as that of siRNA, and cleaves the mRNA of the target gene at a location corresponding to the center of siRNA by RNaseIII-like enzyme activity. Besides this pathway, the antisense strand of siRNA binds to mRNA and acts as a primer for RNA-dependent RNA polymerase (RsRP) to synthesize dsRNA. One may also consider a pathway in which this dsRNA becomes the substrate of Dicer again, to produce a new siRNA to amplify its action.

The above-mentioned RNAi was initially found in *Caenorhabditis elegans* (Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811, (1998)) and has now been observed not only in *C. elegans* but also in various organisms such as plants, nematodes, fruit flies, and protozoans (Fire, A. RNA-triggered gene silencing. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., Caudy, A. A. & Hannon, G. J. Post-transcriptional gene silencing by double-stranded RNA. Nature Rev. Genet. 2, 110-1119 (2001); Zamore, P. D. RNA interference: listening to the sound of silence. Nat. Struct. Biol. 8, 746-750 (2001)). The actual introduction of dsRNA from the outside confirmed that expression of the target gene can be suppressed in these organisms, and this is also being utilized as a method for producing knockout individuals.

Initially when RNAi was introduced, the notion was that dsRNA had to be of a certain length (40 nucleotides) or longer for it to be effective. However, Tuschl *et al.* (Rockefeller University, U.S.A.) reported that by introducing 21 base pairs or so of short chain dsRNA (siRNA) into cells, the RNAi effect was observed, even in mammalian cells, without causing antiviral response due to PKR (Tuschl, Nature, 411, 494-498 (2001)), and RNAi suddenly received attention as a technique applicable to differentiated mammalian cells such as that of humans.

The DNA of this invention comprises an antisense-encoding DNA that encodes an antisense RNA against any one of the regions of the target gene mRNA, and a sense-encoding DNA that encodes a sense RNA of any one of the regions of the target gene mRNA, and the antisense RNA and the sense RNA can be expressed from the antisense-encoding DNA and the sense-encoding DNA. dsRNA can also be prepared from these antisense RNA and sense RNA.

When the dsRNA expression system of this invention is incorporated into a vector or the like, the antisense RNA and sense RNA may be expressed from the same vector, or they may be expressed from different vectors. For example, antisense RNA and sense RNA can be expressed from the same vector by individually assembling an antisense RNA expression cassette and sense RNA expression cassette in which a promoter that may express a short RNA, such as the polIII system, is linked upstream of the antisense-encoding DNA and sense-encoding

DNA, respectively, and inserting these cassettes into a vector in the same direction or in reverse direction. Furthermore, an expression system that has the antisense-encoding DNA and sense-encoding DNA positioned in opposite directions so that they face each other on different strands can be composed. In this arrangement, a single double-stranded DNA in which the antisense RNA-encoding strand and sense RNA-encoding strand are paired (siRNA-encoding DNA) is provided, and promoters can be furnished on both sides in opposite directions so that antisense RNA and sense RNA can be expressed from each of the strands. In this case, to avoid addition of an unnecessary sequence downstream of the sense RNA or antisense RNA, a terminator is preferably placed at the 3' end of each of the strands (antisense RNA-encoding strand and sense RNA-encoding strand). A sequence of four or more continuous A (adenine) nucleotides can be used as this terminator. Furthermore, in this palindromic expression system, the types of the two promoters are preferably different.

On the other hand, antisense RNA and sense RNA can be expressed from different vectors by, for example, individually assembling an antisense RNA expression cassette and sense RNA expression cassette in which a promoter that may initiate expression of a short RNA, such as the polIII system, is linked upstream of the antisense-encoding DNA and sense-encoding DNA, respectively, and inserting these cassettes into different vectors.

In the RNAi of this invention, siRNA may be used as the dsRNA. The term "siRNA" refers to a double-stranded RNA comprising short chains in a range that does not indicate toxicity within cells, and is not limited to an overall length of 21 to 23 base pairs as reported by Tuschl *et al.* (*supra*), so long as the length is within the range that does not indicate toxicity. For example, the length may be 15 to 49 base pairs, preferably 15 to 35 base pairs, and more preferably 21 to 30 base pairs. Alternatively, the siRNA to be expressed may be transcribed such that the final length of the double-stranded RNA portion may be, for example, 15 to 49 base pairs, preferably 15 to 35 base pairs, and more preferably 21 to 30 base pairs.

A construct that forms a double-stranded RNA having a hairpin structure (self-complementary 'hairpin' RNA (hpRNA)) upon insertion of an appropriate sequence (preferably an intron sequence) between the inverted repeats of the target sequence (Smith, N.A. *et al.* Nature, 407:319, 2000; Wesley, S.V. *et al.* Plant J. 27:581, 2001; Piccin, A. *et al.* Nucleic Acids Res. 29:E55, 2001), may also be used as the DNA of this invention.

The DNA used for RNAi does not have to be completely identical to the target gene, but should have a sequence identity of at least 70% or more, preferably 80% or more, more preferably 90% or more, and most preferably 95% or more. The sequence identity can be determined by the methods described above.

In dsRNA, the double-stranded portion in which the RNAs are paired is not limited to those that are completely paired, and it may include unpaired portions caused by mismatches

(wherein the corresponding nucleotides are not complementary), bulges (wherein one of the strands lacks corresponding nucleotides), and such. In the present invention, the double-stranded RNA region in which the RNAs of dsRNA are paired may include both bulges and mismatches.

Endogenous gene expression can also be repressed by cosuppression through the transformation by DNA having a sequence identical or similar to the target gene sequence. "Cosuppression" refers to the phenomenon whereby a gene having a sequence identical or similar to the target endogenous gene sequence is introduced into plants by transformation and expression of both the introduced exogenous gene and the target endogenous gene becomes repressed. Although the detailed mechanism of cosuppression is unknown, it is frequently observed in plants (Curr. Biol. 7: R793, 1997, Curr. Biol. 6: 810, 1996). For example, if one wishes to obtain a plant body in which the CKX gene is cosuppressed, the plant in question can be transformed with a vector DNA designed so as to express the CKX gene or a DNA having a similar sequence to select a plant having the CKX mutant character, *i.e.*, a plant with reduced photoperiod sensitivity, among the resultant plants. The gene to be used for cosuppression does not need to be completely identical to the target gene, but it should have at least 70% or more sequence identity, preferably 80% or more sequence identity, and more preferably 90% or more (*e.g.* 95%, 96%, 97%, 98%, 99%, or more) sequence identity.

In addition, endogenous gene expression in the present invention can also be repressed by transforming the plant with a gene having the dominant negative phenotype of the target gene. Herein, a gene having the dominant negative phenotype refers to a gene which, when expressed, can eliminate or reduce the activity of the wild type endogenous gene inherent to the plant.

The present invention provides vectors into which the DNA of this invention or a DNA that suppresses the expression of the DNA of this invention has been inserted. In addition to the above-mentioned vectors used to produce recombinant proteins, the vectors of this invention also include vectors for expressing the DNA of this invention or a DNA that suppresses the expression of the DNA of this invention in plant cells in order to produce transformed plants. There are no limitations on the type of vectors used, so long as they contain a promoter sequence that can initiate transcription in plant cells, and a terminator sequence comprising a polyadenylation site required for stabilization of the transcript. Examples include plasmids "pBI121", "pBI221", and "pBI101" (all from Clontech). The vectors used for transformation of plant cells are not particularly limited so long as they can express the inserted gene within the cells. For example, vectors comprising a promoter for performing a constitutive gene expression in plant cells (for example, the 35S promoter of cauliflower mosaic virus), and vectors comprising a promoter that is inductively activated by external stimulus may be used. The term "plant cells" used herein includes various forms of plant cells such as suspension

culture cells, protoplasts, leaf sections, and calli.

The vector of this invention may comprise a promoter for constitutively or inductively expressing the protein of this invention. Examples of promoters for constitutive expression include the 35S promoter of cauliflower mosaic virus (Odell *et al.* 1985 Nature 313:810), actin promoter of rice (Zhang *et al.*1991 Plant Cell 3:1155), and ubiquitin promoter of corn (Cornejo *et al.* 1993 Plant Mol. Biol. 23:567).

Examples of promoters for inductive expression include promoters known to initiate expression due to extrinsic factors, such as infection and invasion of filamentous fungi, bacteria, and viruses, low temperature, high temperature, dryness, ultraviolet irradiation, and spraying of particular compounds. Examples of such promoters include the chitinase gene promoter of rice (Xu *et al.* 1996 Plant Mol. Biol. 30:387) and the tobacco PR protein gene promoter (Ohshima *et al.* 1990 Plant Cell 2:95), which are induced by infection and invasion of filamentous fungi, bacteria, and viruses, the "lip19" gene promoter of rice induced by low temperature (Aguan *et al.* 1993 Mol. Gen Genet. 240:1), the "hsp 80" gene and "hsp 72" gene promoters of rice induced by high temperature (Van Breusegem *et al.* 1994 Planta 193:57), the "rab 16" gene promoter of *Arabidopsis thaliana* induced by dryness (Nundy *et al.*, 1990 Proc. Natl. Acad. Sci. USA 87:1406), chalcone synthase gene promoter of parsley induced by ultraviolet irradiation (Schulze-Lefert *et al.* 1989 EMBO J. 8:651), and the alcohol dehydrogenase gene promoter of corn induced by anaerobic conditions (Walker *et al.*, 1987 Proc. Natl. Acad. Sci. USA 84:6624). In addition, the chitinase gene promoter of rice and PR protein gene promoter of tobacco can also be induced by specific compounds such as salicylic acid, and the "rab 16" can also be induced by the spraying of abscisic acid, a phytohormone.

Furthermore, the present invention provides transformed cells to which a vector of this invention has been introduced. The cells to which a vector of this invention is introduced include, in addition to the above-mentioned cells used for producing recombinant proteins, plant cells for preparing transformed plants. There are no particular limitations on the type of plant cells, and examples are cells of *Arabidopsis thaliana*, rice, corn, potato, and tobacco. The plant cells of this invention include, in addition to cultured cells, cells within the plant, and also protoplasts, shoot primordia, multiple shoots, and hairy roots. A vector can be introduced into plant cells by known methods, such as the polyethylene glycol method, electroporation, *Agrobacterium* mediated transfer, and particle bombardment. Plants can be regenerated from transformed plant cells by known methods depending on the type of the plant cell (Toki *et al.*, (1995) Plant Physiol. 100:1503-1507). For example, transformation and regeneration methods for rice plants include: (1) introducing genes into protoplasts using polyethylene glycol, and regenerating the plant body (suitable for indica rice varieties) (Datta, S.K. (1995) in "Gene Transfer To Plants", Potrykus I and Spangenberg Eds., pp66-74); (2) introducing genes into

protoplasts using electric pulse, and regenerating the plant body (suitable for japonica rice varieties)(Toki *et al.* (1992) Plant Physiol. 100, 1503-1507); (3) introducing genes directly into cells by the particle bombardment, and regenerating the plant body (Christou *et al.* (1991) Bio/Technology, 9: 957-962); and (4) introducing genes using *Agrobacterium*, and regenerating the plant body (Hiei *et al.* (1994) Plant J. 6: 271-282). These methods are already established in the art and are widely used in the technical field of the present invention. Such methods can be suitably used for the present invention.

Plants can be regenerated by redifferentiating transformed plant cells. Methods of redifferentiation differ depending on the type of plant cells, and examples include the method of Fujimura *et al.* (Plant Tissue Culture Lett. 2:74 (1995)) for rice, the methods of Shillito *et al.* (Bio/Technology 7:581 (1989)) and Gorden-Kamm *et al.* (Plant Cell 2:603 (1990)) for corn, the method of Visser *et al.* (Theor. Appl. Genet. 78:594 (1989)) for potato, the method of Nagata and Takebe (Planta 99:12 (1971)) for tobacco, the method of Akama *et al.* (Plant Cell Reports 12:7-11 (1992)) for *Arabidopsis thaliana*, and the method of Dohi *et al.* (Unexamined Published Japanese Patent Application No. (JP-A) Hei 8-89113) for eucalyptus.

Once a transformed plant to which the DNA of the present invention or a DNA that suppresses the expression of the DNA of the present invention has been integrated into its genome is obtained, it is possible to obtain a progeny of the plant by sexual or asexual reproduction. It is also possible to obtain reproductive material (such as seeds, fruits, spikes, tubers, tuberous roots, stubs, calli, and protoplasts) from the plant or a progeny or clone thereof, to mass-produce the plant based on such material. Thus, the present invention includes plant cells to which the DNA of the present invention has been introduced, plants containing these cells, progenies and clones of these plants, as well as reproductive material of the plants, and their progenies and clones.

Plants produced in this manner and whose particle-bearing number has been modified show changes in their particle-bearing number and yield when compared to the wild-type plants. For example, plants in which expression of DNA encoding the CKX protein has been suppressed by the introduction of an antisense DNA and such are expected to show increase in yield due to increase in their particle-bearing number. Use of the method of this invention, can increase the particle-bearing number of rice, which is a useful agricultural crop. The present invention is further beneficial in the development of high-yielding rice varieties.

Furthermore, the present invention provides polynucleotides comprising at least 15 continuous nucleotides, which are complementary to the nucleotide sequence of SEQ ID NO: 1 or 2, or their complementary sequences. Herein, the phrase "complementary sequence" refers to a sequence of the other strand with respect to the sequence of one of the strands of a double-stranded DNA comprising A:T and G:C base pairs. The term "complementary" is not

limited to the case in which a sequence is completely complementary in the region of at least 15 continuous nucleotides, and includes cases in which the nucleotide sequence identity is at least 70%, preferably at least 80%, more preferably 90%, and even more preferably 95% or more. Such DNAs are useful as probes for performing detection or isolation of the DNAs of this invention, and as primers for amplifying the DNAs.

Furthermore, the present invention provides methods of genetic diagnosis for determining the increase and decrease in the particle-bearing number of a plant. The particle-bearing number of a plant is closely related to the yield of the plant, and determining the particle-bearing number of a plant is very important in breeding rice varieties for the purpose of increasing the yield.

In the present invention, the phrase "determining the increase and decrease of the particle-bearing number of a plant" includes not only determining the increase and decrease in the particle-bearing number of the varieties cultivated so far, but also includes determining the increase and decrease of the particle-bearing number of new varieties produced by crossing or genetic recombination techniques.

The method of evaluating the increase and decrease of the particle-bearing number of a plant of this invention is characterized by detecting whether or not the plant has lost the function of the DNA encoding the CKX protein. Whether or not the plant has lost the function of the DNA encoding the CKX protein can be evaluated by detecting the change in the nucleotide sequence of genomic DNA corresponding to the CKX gene.

After the nucleotide sequence of a region of a test plant DNA corresponding to the DNA of this invention is determined directly, the plant is determined to be a variety having a small particle-bearing number when the nucleotide sequence encodes a protein whose deletion of function causes an increase in the particle-bearing number of a plant, or determined to be a variety having a large particle-bearing number when this protein is not encoded.

For example, if a mutation causing a deletion of the function of the rice CKX protein is found in the nucleotide sequence of a test plant DNA, this test plant will be diagnosed as a variety having a large particle-bearing number.

Evaluation of the increase and decrease of the particle-bearing number of a plant by the method of this invention has advantages, for example, when breeding improved varieties by crossing plants. For example, when introduction of a trait that increases the particle-bearing number is not desired, one can avoid crossing with varieties having the trait of increasing the particle-bearing number, and in reverse, when introduction of a trait that increases the particle-bearing number is desired, crossing can be performed with varieties having the trait of increasing the particle-bearing number. It is further effective when selecting desirable individuals from crossed progenies. Determination of the increase and decrease of the

particle-bearing number of plants at the genetic level can be performed more conveniently and reliably compared to determination from the phenotype. Therefore, the method for evaluating the increase and decrease of particle-bearing number of this invention may greatly contribute to the breeding of improved plant varieties.

Brief Description of the Drawings

Fig. 1 shows a set of photographs that depict the phenotype of Koshihikari and Habataki. Koshihikari is shown on the left and Habataki is shown on the right.

Fig. 2 shows the positions of Yielding QTL(YQ) on the chromosome.

Fig. 3 shows a small scale linkage map of Yielding QTL(YQ).

Fig. 4 shows a high-resolution linkage map of Yielding QTL(YQ).

Fig. 5 compares the high-resolution linkage maps of Yielding QTL(YQ) of Koshihikari and Habataki.

Fig. 6 shows a phylogenetic tree of the CKX genes of *Arabidopsis thaliana* and rice.

Fig. 7 compares the sequences of the CKX genes.

Fig. 8 is the continuation of Fig. 7.

Fig. 9 is the continuation of Fig. 8.

Fig. 10 shows all of the CKX gene loci in rice.

Best Mode for Carrying out the Invention

Hereinafter, the present invention is specifically illustrated with reference to Examples, but is not to be construed as being limited thereto.

[Example 1] Selection of test materials and production of a semi-isogenic line

Initially, in order to generate a hybrid population for QTL analysis, varieties that would become the parents of the hybrids were selected. First, the average particle-bearing numbers in several varieties of japonica rice and several varieties of indica rice were investigated, and from these varieties, two varieties showing a clear difference in the particle-bearing number, "Koshihikari" which is a japonica rice, and "Habataki" which is an indica rice, were selected (Fig. 1). To F1 individuals produced by crossing japonica rice "Koshihikari" and indica rice "Habataki", backcrossing using Koshihikari as the repeated parent and self-fertilization were carried out, and 74 varieties of BC2F1, BC2F2, and BC3F2 population were cultivated and developed on the Nagoya University Farm.

By performing a QTL analysis relating to the particle-bearing number using 74 BC2F2 plants, a plurality of QTL that increase the particle-bearing number was detected (Fig. 2). In particular, a QTL (YQ1; Yielding QTL 1) of Habataki, located at approximately 28 cM in the

short arm of chromosome 1 (Fig. 2), was successfully discovered to be very effective in increasing the grain (or seed) number as compared to that of Koshihikari. To verify the presence of YQ1, repeated backcrossing and MAS were used to produce a YQ1 semi-isogenic line (Nil-YQ1: a variety in which the locus of Habataki, the locus at approximately 28 cM in the short arm of chromosome 1, is substituted into the chromosome of Koshihikari). The maximum particle-bearing numbers of Nil-YQ1 and Koshihikari (control) were investigated, and as a result, the presence of QTL(YQ1) was confirmed. Varieties in which the locus at approximately 28 cM in the short arm of chromosome 1 was substituted with that of Habataki increased their particle-bearing number by 50 on average.

[Example 2] QTL analysis

From each of the 74 BC2F1 individuals, DNAs were extracted using the CTAB method, and the genotype of each individual was determined using 93 molecular markers that thoroughly cover all chromosomes. Their self-fertilized progeny, BC2F2, was developed at ten individuals per variety, and from among them, one individual per variety was randomly selected, and after sampling six panicles from each of the selected individuals, the particle-bearing number was investigated for each panicle. Among the six panicles of each variety, the panicle having the largest particle-bearing number was selected, and this number was used as the maximum particle-bearing number. QTL analysis was performed using the Qgene software.

[Example 3] High resolution linkage analysis using a segregating population of YQ

The BC3F2 population was used to investigate the phenotype and genotype (F2 and F3) using molecular markers, and linkage analysis was performed again. The results showed that YQ1 is located at the region between molecular markers 6A and 8A (Fig. 3). As a result of performing high-resolution linkage analysis using a segregating population (12500 individuals) of YQ to specify the region of the YQ1 locus, YQ1 was specified to be a region of approximately 8 Kb positioned between molecular markers 4A9 and 20 (Fig. 4). When gene prediction was performed in this region, a single gene was predicted, and as a result of a homology search, a gene that is highly homologous to CKX (cytokinin oxidase) was found (Fig. 4). When the nucleotide sequence of this CKX gene was determined for Habataki and Koshihikari, differences in the nucleotides were found, and the CKX of Habataki seemed to have lost its function (Fig. 5).

[Example 4] Analysis of CKX genes in the rice genome

When the rice genomic sequence was searched to analyze the CKX gene in rice, eleven CKX genes were found in the rice genome. When a phylogenic tree was constructed for these genes as well as the CKX genes in *Arabidopsis thaliana*, AtCKX2, 3, and 4 of *Arabidopsis*

*thaliana*, and five rice CKX genes (CKX located on Chr.1 25 cM P695A4, CKX located on Chr.127 P419B01 (the present gene), CKX gene located on Chr.6 79 cM OsJ0006A22-GS, and two CKX genes located on Chr.2 32 cM) were found to be very closely related (Fig. 6). When the homologies of these genes were investigated, they were found to be highly homologous at the amino acid level (Figs. 7 to 9). Furthermore, when the locus positions of all CKX genes in rice were confirmed, some of them were found to be located on the YQ regions (Fig. 10).

Industrial Applicability

Deletion of the function of the CKX gene provided by the present invention increases the particle-bearing number of plants. Accordingly, regulating the expression of this DNA, using methods such as the antisense method and ribozyme method, can result in increasing the yield of grain. Since genomic synteny (genetic homology) is very highly conserved among grains, application of the rice CKX gene in breeding grains, such as wheat, barley, and corn, can be expected. Furthermore, since the CKX gene is not limited to grains and is widely distributed among plants, deletion of the CKX gene function may increase the number of flowers and seeds (glumous flowers) in all plants, leading to increased yield.

Furthermore, the present invention provides methods of genetic diagnosis for determining the increase or decrease in the particle-bearing number of a plant. Determination of the increase and decrease of the particle-bearing number of plants at the genetic level can be performed more conveniently and reliably compared to determination from the phenotype. Therefore, the method for evaluating the increase and decrease of particle-bearing number of this invention may greatly contribute to the breeding of improved plant varieties.